AD ______________

Award Number: DAMD17-98-1-8356

TITLE: Inhibition of Mammary Cancer by Citrus Limonoids

PRINCIPAL INVESTIGATOR: Ted Vandenberg, M.D.

CONTRACTING ORGANIZATION: The University of Western Ontario
London, Ontario, Canada N6A 5B8

REPORT DATE: August 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2001	Final (1 Aug 98 - 31 Jul 01)

4. TITLE AND SUBTITLE
Inhibition of Mammary Cancer by Citrus Limonoids

5. FUNDING NUMBERS
DAMD17-98-1-8356

6. AUTHOR(S)
Ted Vandenberg, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Western Ontario
London, Ontario, Canada N6A 5B8

E-Mail: ted.vandenberg@lrcc.on.ca

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Limonoids have been shown to inhibit breast cancer cellular proliferation in estrogen receptor negative (ER-) and positive (ER+) cells. Nude mice were given limonoids in both treatment and adjuvant-based models to observe their effects on the proliferation of established and resected tumours respectively. It was found that limonoids, particularly limonin, slowed tumour growth and was able to prevent or delay the regrowth of resected tumours in these models. Additionally, limonoids were detected in mammary tissue samples of mice from the treatment model experiment, indicating that these compounds are bioavailable and were responsible for the observed results.

To elucidate the mechanisms by which limonoids inhibit cellular proliferation, their influence on the cell cycle and abilities to induce apoptosis were investigated. MDA-MB-435 cells were found to accumulate in the G_2M phase of the cell cycle after 72 hours of incubation with limonoids at their IC_{50} values. However, these compounds were not found to induce apoptosis in ER(-) cells when incubated for 48 hours at levels known to be cytostatic. Therefore, the mechanism by which citrus limonoids inhibit cellular proliferation *in vitro* and *in vivo* is via modulation of the cell cycle

14. SUBJECT TERMS
Breast Cancer; Limonoids; Flavonoids; Citrus

15. NUMBER OF PAGES
44

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

TABLE OF CONTENTS

INTRODUCTION

Diet can affect cancer development in many ways. Although there seems to be a strong correlation between dietary fat intake and cancer development in animal models, the translation of these results to humans has been poor. In fact, studies have shown very little correlation between these factors. A cohort study by Hunter in 1996, demonstrated no association between cancer and fat intake over a 20-45% range [1]. Additionally, a study of 65 Chinese counties by Marshall *et. al* in 1992, yielded similar results [2]. This study was interesting because the rationale behind the hypothesis that dietary fat levels are a main determinant in breast cancer development was based on the international correlation between fat consumption and breast cancer. Where breast cancer occurrences are higher in "fatter" Western cultures compared to "leaner" Eastern societies. This correlation did not account for large differences in dietary habits and social behaviors between low and high fat consuming countries.

The differences between Western and Eastern diets are manifold and include the ingestion of many varieties of fruits, vegetables and protein sources. Cooking practices also differ and can influence the formation of carcinogens from foods. Food micronutrient compositions also differ. Micronutrients are phytochemicals that are by-products of plant metabolism generally considered to have no nutritional value. They have been demonstrated to have beneficial health effects and have been the basis of research concerning naturally derived anti-tumour agents [3-5]. Flavonoids and limonoids are two distinct classes of phytochemicals that have shown great potential in the treatment of various health conditions. The citrus derived forms of these compounds are of particular interest because they are unique to this plant form and are well tolerated by most people. Citrus juices are popular beverages widely known for their pleasant taste and antioxidant properties. They are known that have high vitamin C contents, however the beneficial health effects caused by citrus juices may not solely be due to their vitamin content and may be in part due to the actions of flavonoids and limonoids[6].

Limonoids [fig. 1] have been shown to have anti-cancer activity. Nomilin reduced the incidence of and number of chemically-induced forestomach tumors in mice when given by gavage. Addition of nomilin and limonin to the diet inhibited lung tumor formation in mice and topical application of the limonoids was found to inhibit both the initiation and promotion phases of carcinogenesis in the skin of mice [7-13].

Previously our laboratory showed that citrus flavonoids and limonoids are effective inhibitors of estrogen receptor-negative (ER-) and receptor-positive (ER+) human breast cancer cells in culture. It was also shown that the flavonoids were able to delay tumour establishment and prevent metastasis in a nude mouse mammary-xenograft model where ER-, MDA- MB 435 cells were injected into the mammary fat pads of animals. Moreover, whole juices appeared to have a greater inhibitory effect compared to their constituent flavonoids, likely due to the combined actions of other components such as limonoids, vitamin C and hydroxy cinnamic acids. Therefore, to further investigate this result, nude mice were injected in a mammary fat pad, with either ER- or ER+ (MCF-7) human breast cancer cells that were allowed to establish solid tumours. After this the animals were to be given orange and grapefruit juice in place of drinking water to observe the effects on further tumour growth and metastasis[14-18].

BODY

Actions of Citrus Limonoids on the Prevention of Tumour Growth and their Bioavailabilities in Cells and Animal Tissues

Limonoid ER- Treatment Model (Task 2, SOW): In this experiment, animals were given limonin, nomilin and a limonoid glucoside mixture incorporated into the diet at their maximum tolerated doses. This was done so that the effects of these compounds on the growth and metastasis of established mammary fat pad tumours could be observed. Figure 2 depicts the average weights of animals at the beginning and end of the treatment period. Weight gains and losses were monitored to ensure that all animals remained in good health and that the prescribed treatments had no negative health effects. From this figure, it can be seen that all animals were able to maintain their weights over the course of the limonoid treatments except those in the nomilin group. In the nomilin group, the weight average decreased toward the end of the experiment, however this decline was within acceptable ranges (+/- 2 grams) for animal health monitoring practices for individual animals. Additionally, the premature death of one animal much larger than the remaining others one week before the termination of the experiment also helps to explain why the group average drop below the initial start values.

The tracking of food consumption rates was also used as another means by which animal health was monitored. In table 1 food consumption rates before and after the start of limonoid treatments are given. For the control and glucoside mix groups that food consumption remained relatively constant at approximately 4 and 4.06 grams of diet/day/animal. Food consumption in the limonin and nomilin groups was decreased compared to controls. Limonoids are the bitter components of citrus juices [7-9] and their incorporation into the diet may have made them less palatable to the animals and therefore less diet was consumed by these groups. This may be especially true for the nomilin group where food consumption dropped to ±1.66 g/day/mouse in comparison to controls. Decreased food consumption may have also contributed to the weight losses observed in this group toward the end of the experiment.

The effects of citrus limonoids on tumour growth are depicted in Figure 3. Initially, at the beginning of the limonoid treatments, all groups had an even distribution of tumour sizes (some large and some small). At the end of the experiment, tumours in all groups were greater in size compared to their original dimensions, however the overall increase in the sizes of the LIM and GM treated tumours were less than those in the nomilin and control groups. The size increases were 39.6, 27.93, 63.8 and 31.12 mm^2 for the control, LIM, NOM and GM groups respectively in relation to tumour sizes at the beginning of treatment. These changes correspond to 29.5 % and 21.41 % decreases for LIM and GM final tumour surface areas compared to the control, whereas the NOM group had a 61.11% increase. Examining the rates of tumour development over time supported this observation. Figure 4 represents the average tumour sizes (mm^2) for each group before and after dietary limonoid treatment. During the 3 weeks before the initiation of treatments it can be seen that all tumours increased in size at similar rates. Once limonoid treatment began, a divergence in the rates of tumour development for limonin and glucoside mixture groups compared to controls was seen where these tumours grew more slowly. The tumours in the nomilin group appeared to grow much

faster than controls. This indicates that nomilin incorporated into the diet at its maximum tolerated dose was not effective in a treatment-based model and may have instead stimulated growth. The incidences of tumour lung metastases support this statement.

Figure 5 shows the average occurrence of lung metastases for mice in the limonoid treatment groups. The average number of surface lung metastases for the control group was 8.6. Limonin, nomilin and the glucoside mixture had group averages of 6.7, 11 and 10 respectively indicating that limonin was the most effective at preventing the formation of lung metastases by human breast cancer in a treatment based model. Nomilin tended to have a greater number of lung metastases compared to controls and this helps to confirm its ineffectiveness at treating breast cancer in the nude mouse. Although the glucoside mixture was effective at slowing tumour growth over time it seemed to have a greater average number of lung metastases than the controls. This may mean that their may be a bimodal effect for this compound and its effectiveness in treating breast cancer may be a combination of how far the cancer has progressed, where the tumours are located and the concentration of the compound at the treatment site.

Limonoid ER- Adjuvant Model (Task 3, SOW): This study was used to evaluate the anticancer properties of limonin, nomilin and the limonoid glucoside mixture in an adjuvant model that simulates a post-operative chemotherapeutic treatment regimen [18]. During this experiment, actively growing ER- tumours were excised from mice and new tumour development was monitored in relation to dietary limonoid administration. As in the previous experiment, animal weights and food consumption rates were monitored and used as measures to track overall animal health conditions. It can be seen in Figure 6, from the initial injection of tumour cells through the resection of the growing tumours, their subsequent removal and treatment with limonoids, that all animals in all groups gained weight similarly. Initially, a steady increase in weight was expected since this experiment began when mice were immature (3-4 weeks old) and as they aged, their weights increased. After the commencement of dietary limonoid administration, no significant weight losses were observed in any group and food consumption rates remained near control levels. As in the previous experiment nomilin was the least well tolerated of the limonoids (Table 2); however, decreased food consumption for this group did not result in significant weight losses (Figure 6).

Figure 7 depicts the recurrence of tumour growth in animals treated with limonoids post-operatively. The citrus limonoids that appeared to be most effective at preventing the regrowth of ER- mouse mammary fat pad tumours were LIM and NOM whereas the G.M. seemed to be ineffective. Both the control and G.M. groups have respective tumour recurrences of 54 and 55%. LIM and NOM recurrence values were 33% each, indicating that these limonoids may have prevented the regrowth of tumours after resection. This statement is contradicted by the incidence of lung surface metastases for these groups. Figure 8 show a corrective reversed profile for these compounds where LIM and NOM have greater occurrences of metastases compared to controls. Histological analysis of whole and fixed mouse lung tissues revealed that LIM was the most effective at preventing the development of lung surface metastases. The G.M. and NOM had group averages of 10 and 11 lung metastases per animal respectively.

The contradictions between the lung metastasis and mammary fat pad tumour recurrence data may be reflective of differential activities of the limonoids depending on

the stage of breast cancer advancement. These compounds may prevent tumour growth and establishment during early stages of cancer in solid tumours, however after metastasis their effectiveness may be decreased due to alterations in tumour cell physiology. The bioavailability of these compounds at the site of interest may also be a factor in their relative effectiveness. Lypophyilic compounds may be more readily available to tissue having high fat contents such as mammary tissue, whereas they may have little influence in lung tissues due to lower concentrations. Flavonoids have been demonstrated by several groups [19] to have biphasic effects *in vitro*. Very low media concentrations can stimulate cancer cell growth whereas higher doses strongly inhibit cellular proliferation. Limonoids in this circumstance and in the previous experiment may have acted in a similar manner *in vivo*, however the effective concentrations in tissues by these compounds is yet unknown.

Another possibility may be that limonoids suppress the immune response. Flavonoids have been demonstrated to have immunomodulatory effects and the limonoids may be acting similarly [19]. At resection site these compounds may have acted directly on the remaining cells present, however in the lungs their immunosuppressive abilities may have been responsible for the increased numbers of metastases [20, 21]. It is well known that clearance of lung metastases is dependent on the immune system. Depressed immune function may have allowed metastases in the LIM and NOM groups to form and grow in an uncontrolled manner leading to the observed results.

In addition, surgical tumour removal also leads to decreased immune function with respect to wound healing. During wound healing cortisol levels increase which in turn suppress immune function. At this time, circulating metastatic cells may become lodged in the lungs and their immune clearance impaired. Further immunosuppresion by limonoid treatment at this time may exacerbate this situation resulting in greater metastasis formation [20, 21].

Limonoid ER+ Treatment Model (Task 4, SOW): In this experiment, animals were given limonin, nomilin and a limonoid glucoside mixture incorporated into the diet at their maximum tolerated doses. This was done so that the effects of these compounds on the growth and metastasis of established ER+ mammary fat pad tumours could be observed. Weight gains and losses were monitored to ensure that all animals remained in good health and that the prescribed treatments had no negative health effects (Table 3). The tracking of food consumption rates was also used as another means by which animal health was monitored. It can be seen for the control and glucoside mix groups that food consumption remained relatively constant at approximately 4 and 4.06 grams of diet/day/animal. Food consumption in the limonin and nomilin groups was decreased compared to controls.

The effects of citrus limonoids on ER+ tumour growth are depicted in Table 3. At the end of the experiment, tumours in all groups were greater in size compared to their original dimensions, however the overall increase in the sizes of the NOM and GM treated tumours were less than those in the LIM and control groups (Table 3). These findings are different from what has been observed for the ER- tumors. This indicates that nomilin and the glucoside mixture incorporated into the diet at their maximum tolerated dose was effective in a reducing tumor growth whereas limonin was the least effective. No metastases were observed in this model. The MCF-7 ER+ cells are less aggressive than the ER- cells and do not metastasize to other sites as we observed in in the ER- model.

Limonoid ER+ Adjuvant Model (Task 5, SOW): This study was used to evaluate the anticancer properties of limonin, nomilin and the limonoid glucoside mixture in an adjuvant model that simulates a post-operative chemotherapeutic treatment regimen [17]. During this experiment, actively growing tumours (ER+) were excised from mice and new tumour development was monitored in relation to dietary limonoid administration. As in the previous experiment, animal weights and food consumption rates were monitored and used as measures to track overall animal health conditions (Table 4). The citrus limonoids were ineffective at preventing the regrowth of ER+ mouse mammary fat pad tumours (Table 4).

Limonoid Bioavailability (Task 7, SOW): The bioavailability of citrus limonoids and their metabolites was investigated using extracts of tissues removed from animals in both experiments. Tissues from the mammary glands, kidneys, liver, and spleen were removed, lyophilized, ground and sent for HPLC analysis. Detection of limonoids in mouse tissues by HPLC/MS [16] was observed in the mammary and kidney tissues of the limonoid treatment group. Both samples contained significant amounts of limonin, however quantification was made difficult because a limonin standard curve could not be established due to instability in the solvent system required for mass spectrometry. No known limonoids were detected in the G.M treated group. However, several peaks on the chromatogram were detected that were not present in control samples. These peaks may have been representative of limonin or limonoid glucoside metabolites whose structures remain unknown and were not identifiable by mass spectrometry.

The detection of limonoids in animal tissues indicate that these compounds are absorbed when consumed in the diet and that they are able to reach the circulation. The detection of limonin in the mammary tissues and kidneys of the extracted samples also demonstrates that these compounds are bioavailable and that they reached the target breast tissue for this experiment. Limonin is an extremely lypophylic molecule and therefore is water insoluble. Its presence in the mammary tissue is expected since this tissue has a high fat content and therefore would be more accessible to lypophylic compounds. The accumulation of limonin in the mammary tissues before tumour cell injections may have inhibited cellular proliferation thus discouraging solid tumour formation.

Studies on the Mechanism of Action of Citrus Limonoids (Task 6, SOW)

Induction of Apoptosis: To determine if citrus limonoids are inducers of apoptosis, MDA cells were incubated with the compounds at the concentrations listed in Table 5. Actinomycin D (A.D.) was used as a positive control for the staining procedure whereas since it induces apoptosis in HL-60 leukemia cells. Table 5 shows the percentages of cells stained by annexin and or P.I. in the *Vybrant apoptosis assay*. Annexin would positively stain cells in early apoptotic stages whereas cells in later stages would be doubly stained for both markers.

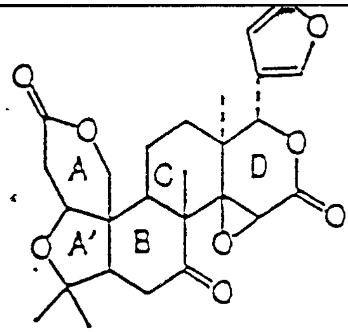
It can be seen that the positive control A.D. strongly induced apoptosis in cells where approximately 60% of cells were stained by annexin. For all other compounds tested, no significant changes in the percent of cells stained positively for annexin were observed over untreated controls. This indicates that these citrus limonoids do no mediate their effects through the induction of apoptosis at the concentrations used.

Cell Cycle Analysis: The modulation of the cell cycle by citrus limonoids was examined by flow cytometry. The incorporation of the DNA intercalating agent BrdU was used to measure the DNA content of individual cells when bound by an anti-BrdU antibody conjugated to FITC. MDA-MB-435 cells were incubated with limonoids at their IC_{50}'s. Figure 9 is a depiction of the cell cycle distribution given by the Modfit program used to analyze data obtained with the flow cytometer. The hatched regions represent S-phase DNA content whereas the solid peaks to the left and right of this region represent DNA in the G_0/G_1 or G_2M phases respectively. The images presented are representative of data acquired in a typical experiment performed on the same day for the citrus limonoids and their controls whereas Figure 10 is a representation of the cell cycle distribution for this group over 5 separate experiments. It can be seen in 10(c) that the percentage of cells in the G_2M phase is greatly increased over untreated and DMSO controls. Corresponding decreases in the G_0/G_1 and S phase DNA contents are observed in 10(a) and 10(b).

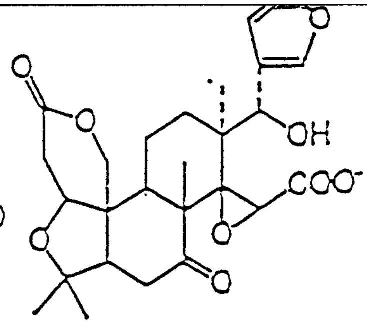
For all experiments genistein was used as a positive control since this soy isoflavone has been shown to cause accumulation in either the G_0/G_1 or G_2M phases depending on the cell line used. Here, genistein caused accumulation in the G_2M phase at an average level of 350% above controls. The citrus limonoids were much more potent inducers of cell cycle arrest. Limonin was the strongest inducer where a 550% increase in the number of cells in the G_2M phase was observed. The least potent limonoid was the G.M. with only a 260% increase in cells having DNA in the G_2M phase whereas nomilin had an intermediate value of 448% above controls. These experiments demonstrate that citrus limonoids are inducers of cell cycle arrest in the G_2M phase in MDA-MB-435 cells and they do so within a range of 99-550% above controls.

The order of potency observed for the limonoids have a reversed profile when compared to proliferation assays. Here, limonin was the strongest inducer of cell cycle arrest followed by nomilin and the G.M., but in proliferation assays the G.M was the strongest inhibitor and limonin the weakest. The mechanisms by which these compounds induce this arrest must be further elucidated to fully understand which aspect of the cell cycle machinery each compound affects.

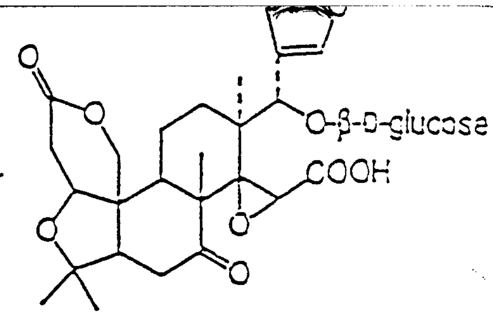
P-Glycoprotein Interactions: The interaction of P-glycoprotein (P-gp) with limonoids was tested by co-incubation of MDA-MB-435 cells with Verapamil (a P-gp inhibitor) and these citrus juice components. Cells were extracted and a comparison between cells given limonoids in the absence or presence of Verapamil was drawn. A positive interaction with P-gp would be indicated by an increase in the extract concentrations, resulting due to increased competition for P-gp binding sites between the limonoids and verapamil. When cells were incubated with limonin and nomilin at 10μg/ml, extracts had no detectable levels of these compounds and therefore their interactions with P-gp were not evaluated. This may have been due to the fact that limonin and nomilin are extremely insoluble in aqueous media and may have precipitated out of solution. When the cells were washed before extraction nomilin and limonin would have been removed from the extracellular environment. They still remain effective both *in vitro* and *in vivo* since their negative effects on cellular proliferation have been established [17] and they were shown to slow the growth of mammary cancer in the nude mouse model described above. This indicates that their effects may be mediated by mechanisms other than interaction with P-gp that are currently unknown.



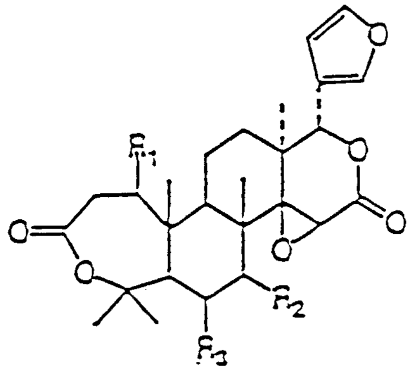
Limonin (1)



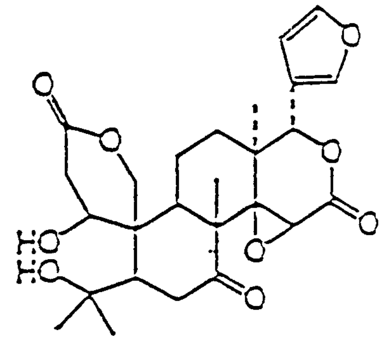
Limonoate A-ring lactone (2)



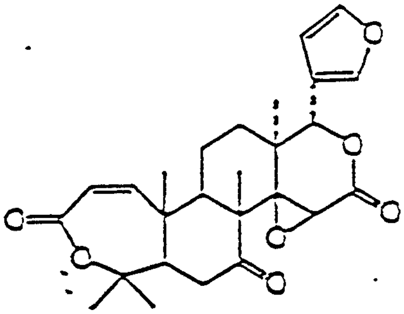
Limonin 17-β-D-glucopyranoside (3)



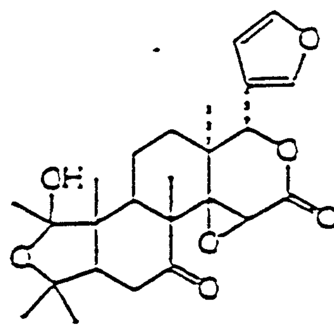
Nomilin (4) R_1=OAc, R_2=O, R_3=H
Deacetylnomilin (5) R_1=OH, R_2=O, R_3=H
6-keto-7β-Deacetylnomilol (6) R_1=OH, R_2=OH, R_3=O



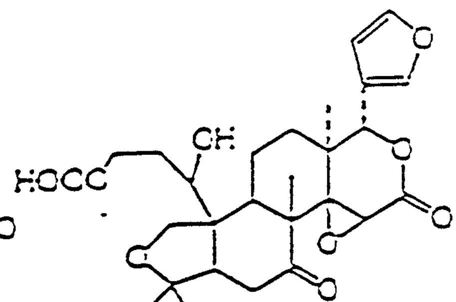
Ichangin (7)



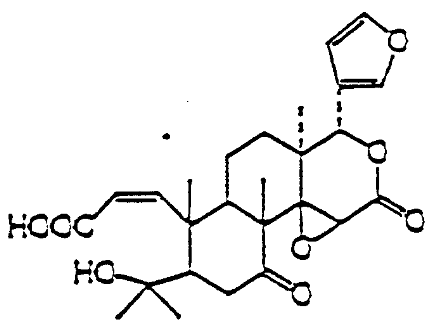
Obacunone (8)



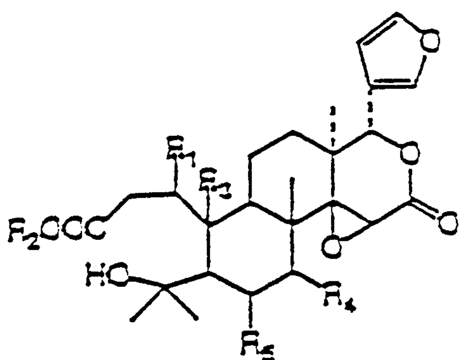
Ichangesin (9)



Isolimonic acid (10)



Obacunoic acid (11)



Nomilinic acid (12) R_1=OAc, R_2=H, R_3=CH_3, R_4=O, R_5=H
Deacetylnomilinic acid (13) R_1=OH, R_2=H, R_3=CH_3, R_4=O, R_5=H
Calamin (14) R_1=OH, R_2=CH_3, R_3=CH_3, R_4=OH, R_5=O
19-Hydroxydeacetylnomilinic acid (15)
R_1=OH, R_2=H, R_3=CH_2OH, R_4=O, R_5=H

Fig. 1: Structures of Major Limonoids in Citrus
(Reproduced from Hasagawa et al., Biochemistry of Citrus Limonoids)

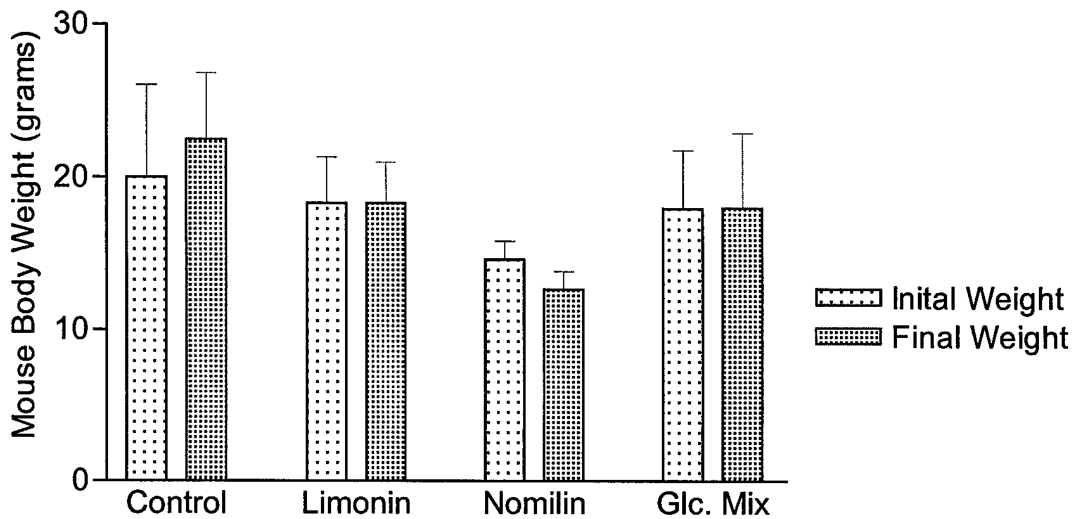


Figure 2: Initial Versus Final Body Weights for Animals Given Citrus Limonoids in a Treatment-Based Model. Results are presented as group averages ± S.E.M.

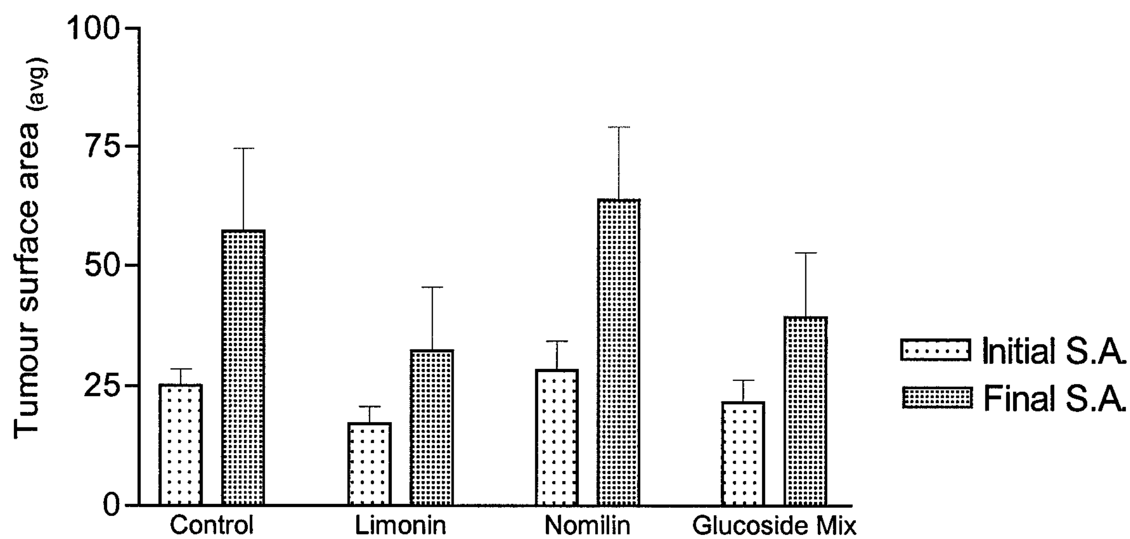


Figure 3: Tumour Surface Areas (mm^2) for Nude Mice Given Limonin, Nomilin and a Limonoid Glucoside Mixture in the Diet at 1%, 0.5% and 1% Respectively at the Beginning and End of the Treatment Period. Results are presented as group averages ± S.E.M.

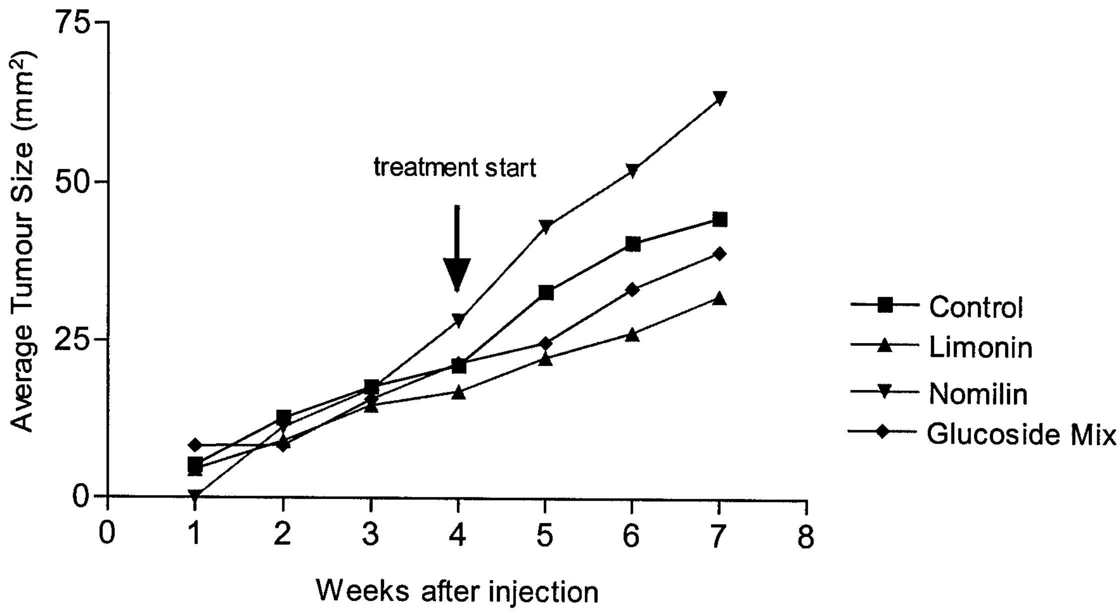


Figure 4: The Development of Mammary Fat Pad Tumours in Mice Given Citrus Limonoids in the Diet with Respect to Time (Weeks).

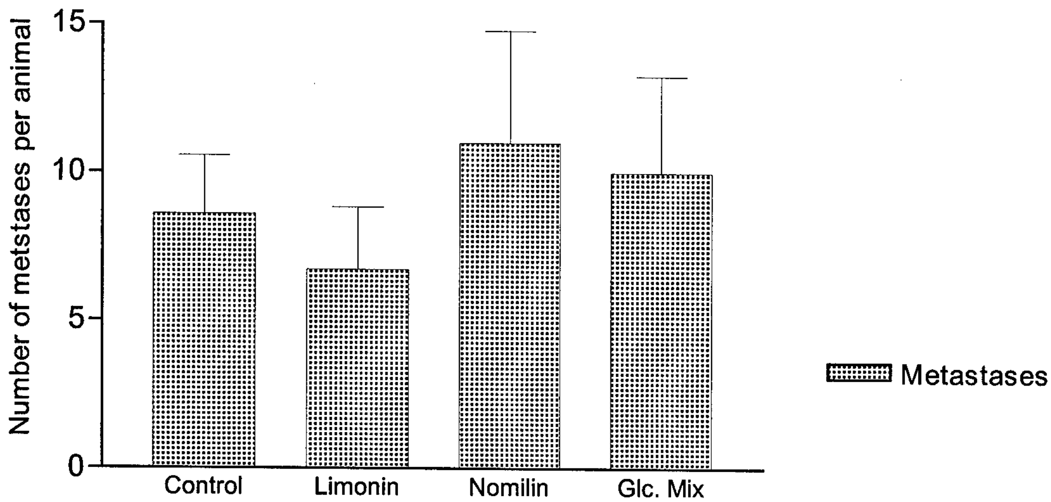


Figure 5: The Average Incidence of Lung Surface Metastases in Nude Mice Treated with Limonin, Nomilin and a Limonoid Glucoside Mixture in a Treatment-Based Model. Results are presented as group averages ± S.E.M.

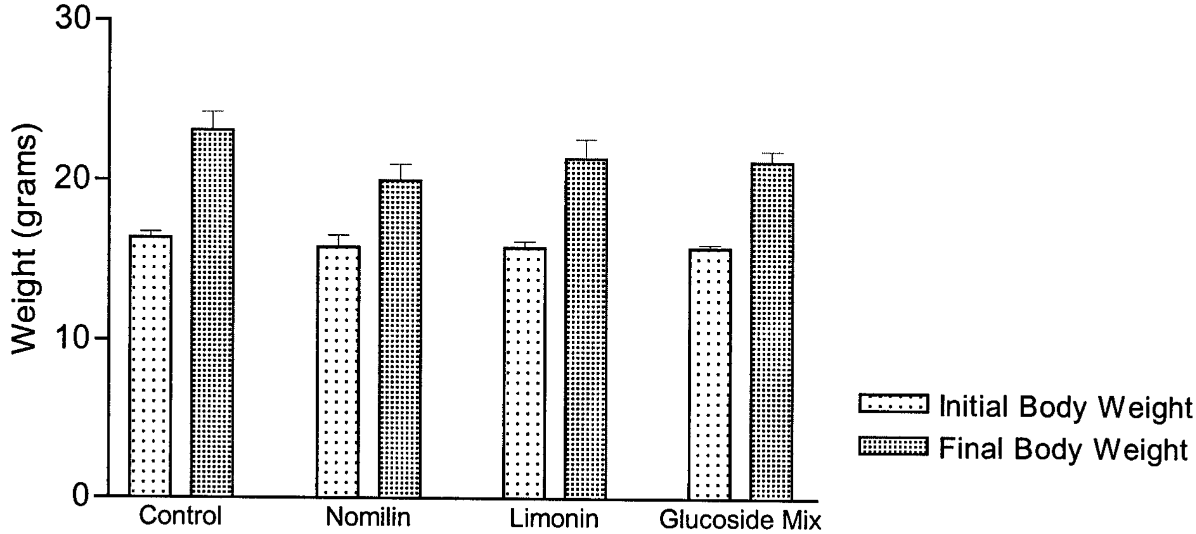


Figure 6: Animal Weights for Dietary Treatment Groups at Beginning and End of the Limonoid Adjuvant Study. Results are presented as group averages ± S.E.M.

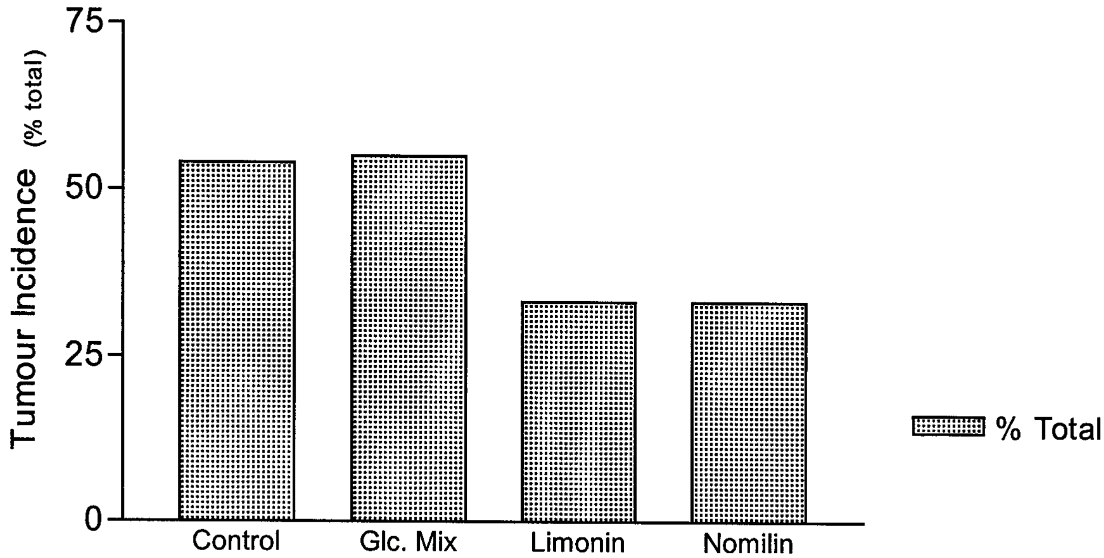


Figure 7: Incidence of Mammary Fat Pad Tumour Recurrence in Nude Mice Given Citrus Limonoids in the Diet after Primary Tumour Resection. Results are present as the percent of group total.

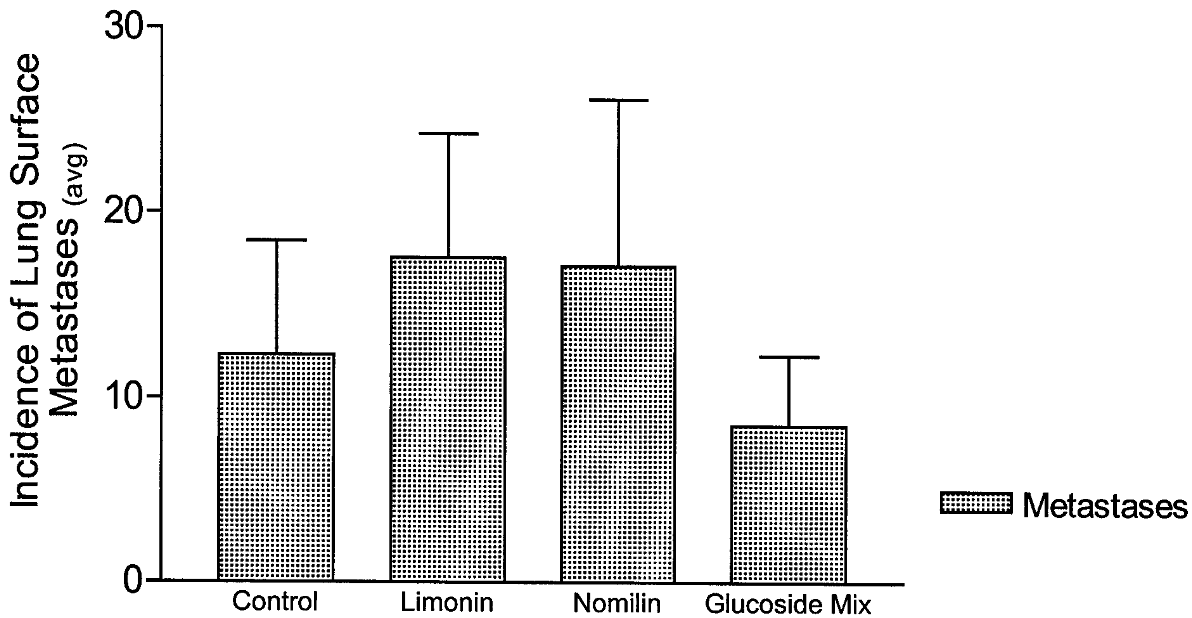
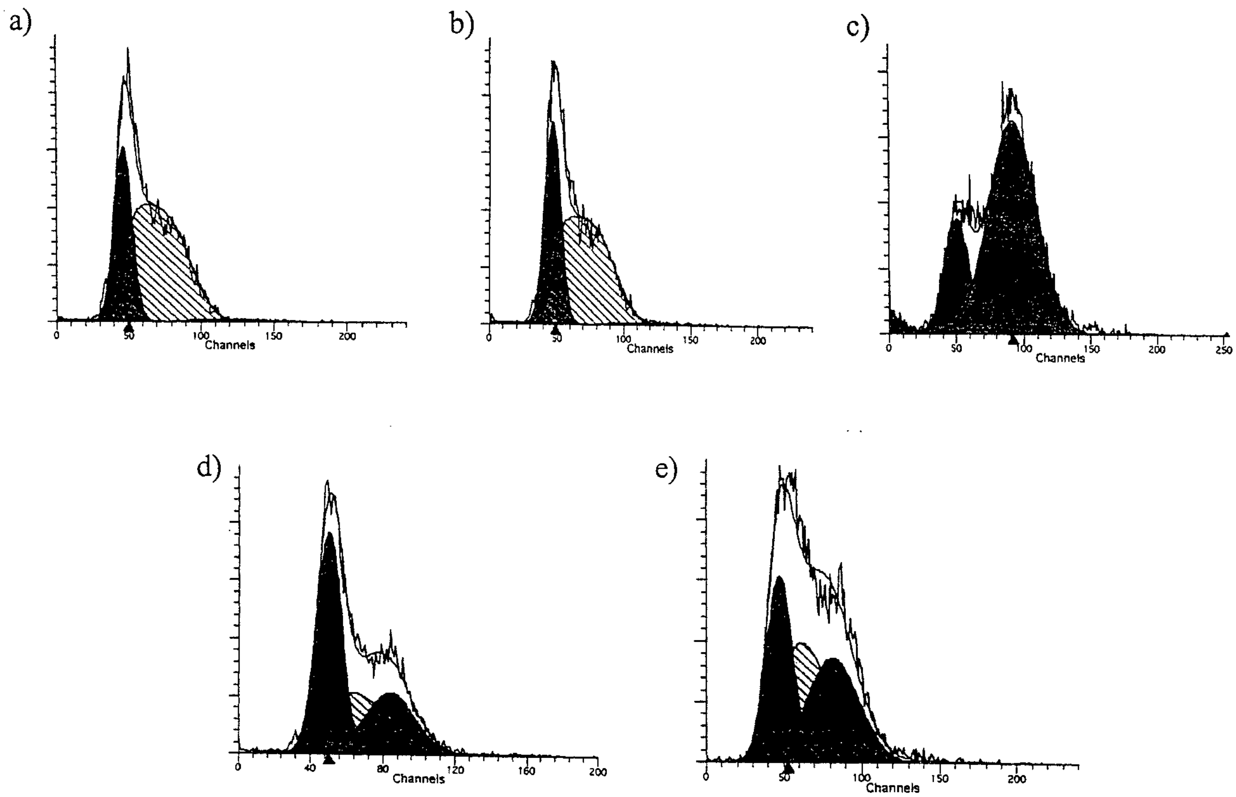


Figure 8: The Incidence of Lung Surface Metastases in Nude Mice Given Dietary Citrus Limonoids in an Adjuvant-Based Model. Results are presented as group averages ± S.D.



<u>Figure 9</u>: Modfit Cell Cycle Graphical Representations of the Cell Cycle Distribution of MDA-MB-435 Cells Treated with Citrus Flavonones at their IC_{50}'s in One Experiment. a) Untreated Negative Control, b) DMSO Vehicle Control, c) Genistein Positive Control, d) Glucoside Mixture and e) Limonin.

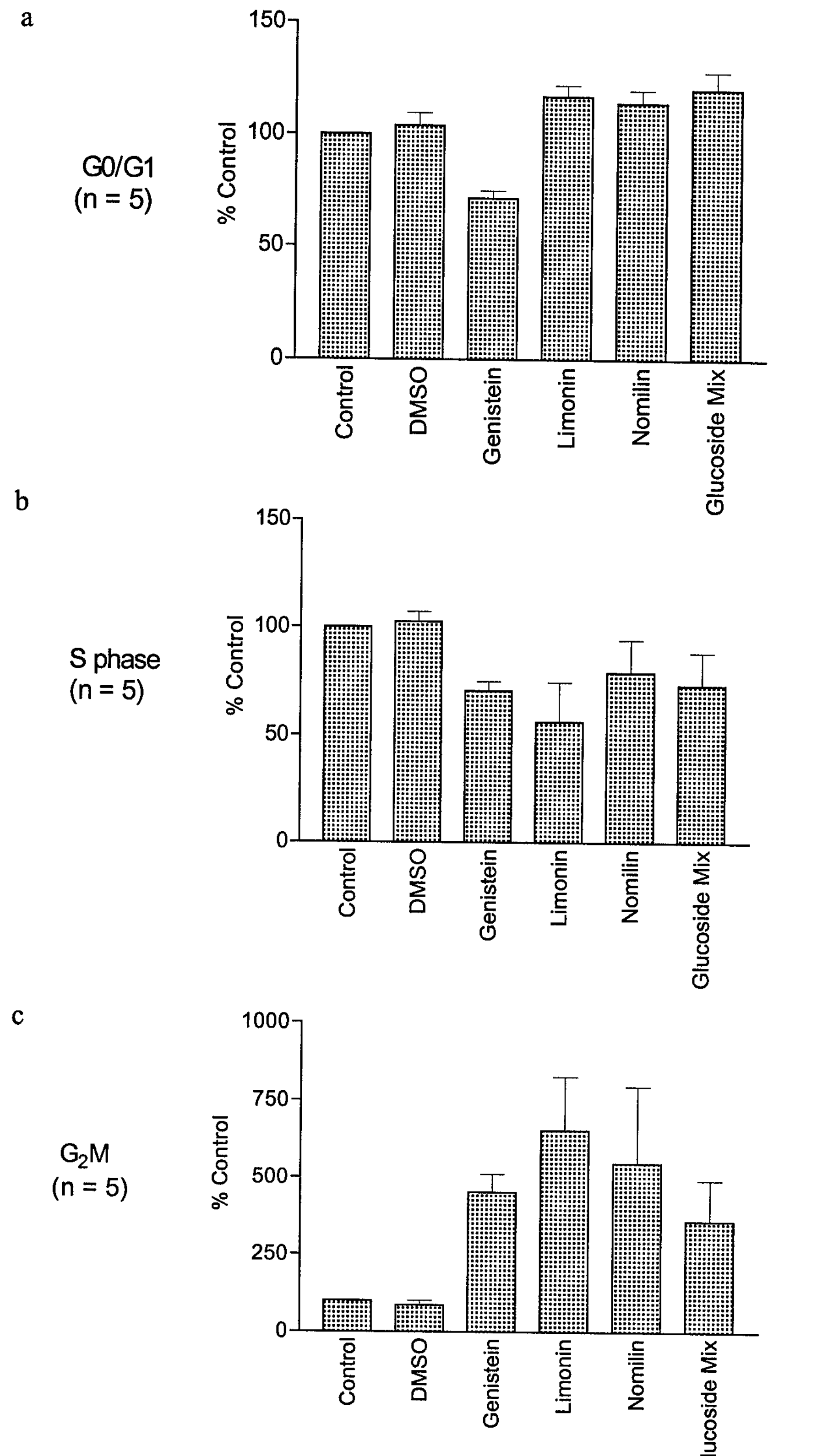


Figure 10: The Cell Cycle Distribution of MDA-MB-435 Cells Treated with the Citrus Limonoids. a) G_0/G_1, b) S Phase, c) G_2M.

Table 1

Average Food Consumption for Mice Given Citrus Limonoids in a Treatment Based Cancer Model.

Experimental Group	***Food Consumption*** ***average*** ***g/day/mouse***
Control	4.0
Glucoside Mix	4.07 ± 0.95
Limonin	3.13 ± 1.48
Nomilin	1.66 ± 0.47

(Results are given as group averages ± S.D.)

Table 2: Food consumption for Mice Treated with Citrus Limonoids in an Adjuvant Model. Results are presented as group averages ± S.D.

Control	3.32 ± 0.84
Glucoside Mix	3.64 ± 1.11
Limonin	3.54 ± 0.63
Nomilin	3.04 ± 0.83

Table 3

Effect of Citrus Limonoids on the Incidence and Growth of MCF-7 Estrogen-Receptor–Positive Human Breast Cancer Cells in Nude Mice

Cell Line	Diet	No. of Mice	Food Consumpt. G/mouse/d	Initial Weight (g)	Final Weight (g)	Initial Tumor Incidence	Initial Tumor Surf. Area (cm^2)	Final Tumor Surf. Area (cm^2)	Tumor Weight (g)
MDA	Control	12	4	14.7±1.2	13.3±3.1	100%	0.418±0.02	1.47±0.19	0.58±0.10
	Lim 2%	12	3.1	16.7±1.2	14.7±1.2	100%	0.409±0.11	1.17±0.16	0.46±0.10
	GM 2%	12	4.4	16.7±2.3	14.7±4.2	100%	0.393±0.05	0.69±0.23	0.20±0.09
	Lim 1%	12	1.7	14.7±1.2	12.7±1.2	100%	0.422±0.06	0.89±0.23	0.28±0.04

Table 4: Effect of Citrus Limonoids on the Recurrence of MCF-7 Estrogen-Receptor-Positive Human Breast Cancer Cells in Nude Mice.

Group	Initial Weight (g)	Final Weight (g)	Growth Rate (g/day)	Food Consumpt. g/mouse/d	Tumor Incidence Before Removal (%)	Tumor Size (cm^2) Before Removal	Tumor Weight After Removal (g)	Tumor Recurrence After 7 Weeks on Diets (%)	Tumor Size (cm^2) recurrence after 7wks on Diets	Tumor Weight(g) Recurrence After 7 wks on Diets
Control	20.44± 2.79	21.56± 3.43	0.026± 0.041	3.02±0.62	78	0.203± 0.202	0.096± 0.112	22	0.084± 0.184	0.033± 0.071
0.5% Nom	18.89± 2.26	21.11± 2.03	0.052± 0.036	2.89±0.31	78	0.204± 0.178	0.066± 0.069	33	0.056± 0.094	0.013± 0.033
1% Lim	18.5± 2.33	20.00± 2.14	0.035± 0.041	3.31±0.96	63	0.175± 0.223	0.075± 0.101	25	0.026± 0.061	0.001± 0.004
1% G.M.	18.67± 1.00	22.22± 1.56	0.083± 0.031	3.56±0.23	78	0.201± 0.180	0.084± 0.078	22	0.031± 0.061	0.007± 0.013

Table 5: The induction of apoptosis in MDA-MB-435 human breast cancer cells by citrus limonoids determined by annexin-V and propidium iodide staining. The result presented are the averages of 5 separate determinations ± S.E.M.

Treatment	Concentration (μg/ml)	% PI Stained	% PI + Annexin Stained	%Viable	% Annexin Stained
No treatment	0	0.23	4.62	92.6	2.55
Actinomycin D	0.08	4.53	44.38	35.92	15.16
Limonin	35	1.51	9.46	86.04	2.98
Nomilin	10	1.19	10.03	82.47	6.13
Glucoside Mix	10	6.13	15.04	73.8	4.13

KEY RESEARCH ACCOMPLISHMENTS

- Two hundred grams of each of the limonoids were isolated.
- Effect of citrus limonoids on treatment of ER- tumors in nude mice was investigated at their maximum tolerated dose.
- Effect of citrus limonoids on ER- tumor recurrence in nude mice was investigated.
- Effect of citrus limonoids on treatment of ER+ tumors in nude mice was investigated at their maximum tolerated dose.
- Effect of citrus limonoids on ER+ tumor recurrence in nude mice was investigated.
- Studies investigating the mechanism of action of limonoids were completed.
- Citrus limonoids were measured in the mammary fat pads of nude mice.
- Presented data at Canadian Breast Cancer Research Initiative conference, Toronto, ON, November 2000.
- Presented data at ISF Congress, Orlando, FL, December, 2000.
- Presented data at Breast Cancer meeting, San Antonio, TX, December, 2000.

REPORTABLE OUTCOMES

PUBLICATIONS AND MEETING ABSTRACTS

1. Guthrie, N., Hasegawa, S., Manners, G. & Carroll, K.K. Inhibition of human breast cancer cells by citrus limonoids. Proc. Annual Meeting, Am. Chem. Soc., Limonoid Symposium, Anaheim, CA, March 22-24, 1999 (Abstract).

2. White, Dionne, Guthrie, N., Freeman, D. & Vandenberg, T.A. Inhibition of Mammary tumorigenesis by citrus juices. Proc. Can. Breast Cancer Res. Intiative, Reasons for Hope, Toronto, ON, June 17-19, 1999 (Abstract).

3. Morley, K. & Guthrie, N. Inhibition of estrogen receptor-negative breast cancer by citrus limonoids (4th year student research project).

4. Vandenberg, T., White, D. & Guthrie, N. The effectiveness of citrus juice components on human breast cancer cell proliferation, Proceedings of Cancer Care Ontario Conference on Hormone Sensitive Cancer, Cochiching, ON, Nov. 9-11, 1999 (abstract).

5. Guthrie, N., Morley, K., Hasegawa, S., Manners, G.D. & Vandenberg, T.A. Inhibition of Human Breast Cancer Cells by Citrus Limonoids. In: Citrus Limonoids:Functional Chemicals in Agriculture and Food. M.A. Berhow , S. Hasegawa, G.D. Manners (eds.), American Chemical Society, Washington, DC, pp. 164-174, 2000.

The US Army Medical and Materiel Command under DAMD17-98-1-8356 supported this work

PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

Najla Guthrie, Carina Banh, Charlotte Harman, Karen Morley, Leanne Reid, and Dionne White

CONCLUSIONS

The data presented in this report demonstrates that using the nude mouse model, the citrus limonoids were well tolerated. In the treatment model, limonoids, especially Limonin inhibited the growth of tumours compared to control in ER- tumors and that NOM and GM were more effective in ER+ tumors. Lung metastasis still developed although it is possible the rate of development may be slowed by limonin. Regrowth of resected mammary fat pad tumours may be reduced by limonin and nomilin but more rigorous assessment of other factors that may contribute to local recurrence and confirmatory studies are required. Limonoids may have differential effects on local recurrence and lung metastases. This observation requires confirmation and further investigation if replicated. There seems to be a differential effect of citrus limonoids on on ER+ and on ER- tumours using MCF-7 and MDA-MB435 cell lines. Anti-tumour effects of citrus limonoids are not mediated via apoptosis. Citrus limonoids have both cell cycle arrest anti-proliferation effect but the relative potency is different for each limonoid. Unable to evaluate interaction with p-glycoprotein.

Overall the use of citrus juice components in the prevention of mammary cancer may be beneficial. They are bioavailable where LIM was detected in the mammary tissue of mice and hesperetin was detected in the liver. Future work into these compounds should be focused upon pinpointing which cell cycle components they affect and signal transduction pathways they disrupt. Clarification of these aspects would provide more information into their mechanisms of action and provide insight as to how they may be specifically exploited and targeted in breast cancer treatment. Their effects on the immune system should also be investigated. Determination of the immune cells affected by citrus limonoids and their subsequent cytokine release would be useful in further isolating their activities and defining their role as anticancer agents with respect to the immune response. This would be especially important to physicians where immunomodulators are often used as chemotherapeutic agents.

REFERENCES

1. Hunter DJ, Spiegelman D, Adami HO, Beeson L, van den Brandt PA, Folsom AR, Fraser GE, Goldbohm RA, Graham S, and Howe GR (1996). Cohort studies of fat intake and the risk of breast cancer--a pooled analysis. *N Engl J Med* **334(6),** pp. 356-361.
2. Marshall JR, Qu Y, Chen J, Parpia B, Campbell TC (1992). *Additional ecological evidence: lipids and breast cancer mortality among women aged 55 and over in China.* Eur J Cancer. **28A(10),** pp. 1720-7.
3. Willet, W.C. (1997) Fat, energy and breast cancer. *Journal of Nutrition*, **127**, pp. 921s-923s.
4. Block, G., Patterson,B., and Subar, A. (1992) Fruit, vegetables and cancer prevention: A review of the epidemiological evidence. *Nutrition and Cancer*, **18**, pp. 1-29.
5. Hollman, P.C.H., and Katan, M.B. (1997) Absorption, metabolism and health effects of dietary flavonoids in man. *Biomedicine and Pharmacotherapeutics*, **51**, pp. 305-310.
6. So, F.V., Guthrie, N., Chambers, A.F., Moussa, M., and Carroll, K.K. (1996) Proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutrition and Cancer*, **26**, pp. 167-181.
7. Hasegawa, S., Bernhow, M.A., and Manners, G.D. (2000) Citrus Limonoid Research: An overview, *In: Citrus Limonoids; Functional Chemicals in Agriculture and Foods.* Bernhow, M.A., Hasegawa, S.H., and Manners, G.D., eds., © American Chemical Society, pp.3-7.
8. Hasegawa, S. (2000) Biochemistry of Limonoids in Citrus. *In: Citrus Limonoids; Functional Chemicals in Agriculture and Foods.* Bernhow, M.A., Hasegawa, S.H., and Manners, G.D., eds., © American Chemical Society, pp. 9-29.
9. Miller, E.G., Fanous, R., Rivera-Hidalgo, F., Binnie, W.H., Hasegawa, S., and Lam, L. K.T. (1989) The effect of citrus limonoids on hamster buccal pouch carcinogenesis. *Carcinogenesis*, **10(8)**, pp. 1535-1537.
10. Lam, L.K.T., and Hasegawa, S. (1989) Inhibition of benzo[a]pyrene-induced forestomach neoplasias in mice by citrus limonoids. *Nutrition and Cancer*, **12**, pp. 43-47.
11. Lam, K.T., Hasegawa, S., Bergstrom, C., Lam, S.H., and Kenney, P. (2000) Limonin and Nomilin Inhibitory Effects on Chemically-Induced Tumorigenesis. *In: Citrus Limonoids; Functional Chemicals in Agriculture and Foods.* Bernhow, M.A., Hasegawa, S.H., and Manners, G.D., eds., © American Chemical Society, pp. 185-199.
12. Tanaka, T., Kohno, H., Kawabata, K., Honjo, S., Miyake, M., and Wada, K. (2000) Citrus Limonoids Obacunone and Limonin Inhibit the Development of a Precursor Lesion, Aberrant Crypt Foci, for Colon Cancer in Rats. *In: Citrus Limonoids; Functional Chemicals in Agriculture and Foods.* Bernhow, M.A., Hasegawa, S.H., and Manners, G.D., eds. © American Chemical Society, pp 145-163.
13. Lam, L.K.T., Sparnins, V.L., and Wattenberg, L.W. (1987) Effects of derivatives of kahweol and cafestol on the activity of glutathione S-transferase in mice., *Journal of Medicinal Chemistry*, **30**, pp. 1399-1403.

14. Guthrie, N., and Carroll, K.K. (1998) Inhibition of Human Breast Cancer Cell Growth and Metastasis in Nude Mice by Citrus Juices and their Constituent Flavonoids, *In: Biological Oxidants and Antioxidants: Molecular Mechanisms and Health Effects*. Packer, L., and Ong, A.S.H., eds., AOCS Press, Champaign, IL., pp. 310-316.
15. So, F.V, Guthrie, N., Chambers, A.F., and Carroll, K.K. (1997) Inhibition of estrogen receptor-positive MCF-7 human breast cancer cells by flavonoids in the presence and absence of excess estrogen. *Cancer Letters*, **112**, pp. 127-133.
16. Guthrie, N., and Carroll, K.K., (1998), Inhibition of Mammary Cancer by Citrus Flavonoids, *In: Advances in Experimental Biology: Flavonoids in the Living System*, **439**, Manthey, J., and Buslig,B., Eds. Plenum Press, New York, NY., pp. 227-236.
17. Guthrie, N., Morley, K L., Hasegawa, S.H., Manners, G.D., and Vandenberg, T. (2000) Inhibition of Human Breast Cancer Cells by Citrus Limonoids *In: Citrus Limonoids; Functional Chemicals in Agriculture and Foods.* Bernhow, M.A., Hasegawa, S.H., and Manners, G.D., eds., © American Chemical Society, pp. 164-174.
18. Sledge, G.W., Qulalo, M., Goulet, R., Bone, E., and Fife, R. (1995), Effect of matrix metalloproteinase inhibitor batimastat on breast cancer regrowth and metastasis in athymic mice. *Journal of the National Cancer Institute*, **87(20)**, pp. 1546-1150.
19. Manthey, J.A, Grohmann, K., and Guthrie, N., (2001). Biological properties of citrus flavonoids pertaining to cancer and inflammation. *Current Medicinal Chemistry*, **8(2)**, pp. 135-53.
20. Pollock, R.E. Lotzova, E., and Stanford,S.D, (1989), Surgical stress impairment of murine natural killer cell cytotoxicity involves pre- and postbinding events. *Journal of Immunology,* **143(10)**, pp. 3396-3403.
21. Ogawa, K., Hirai, M., Katsube, T., Murayama ,M., Hamaguchi ,K., Shimakawa, T., Naritake, Y., Hosokawa, T., Kajiwara, T. (2000), Suppression of cellular immunity by surgical stress. *Surgery*, **127(3)**, pp. 329-36.
22. Manners, G.D., and Hasegawa, S. (1999) A new normal phase liquid chromatographic method for the analysis of limonoids in citrus. *Phytochemical Analysis*, **10**, pp. 76-81.

APPENDICES

American Chemical Society Meeting
Citrus Limonoids Functional Chemicals in Agriculture and Foods
Anaheim, CA
Mar. 21st-25th, 1999.

. . . .

Inhibition of human breast cancer cells by citrus limonoids.

Guthrie, N., Hasegawa, S., Manners, G.D. and Carroll, K.K.

Citrus limonoids are a class of chemically related compounds present in lemon, lime, orange and grapefruit (Hasegawa *et al.*, 1994). We have shown that nomilin and a limonoid glucoside mixture are potent inhibitors of proliferation of estrogen receptor-negative (ER-) and -positive (ER+) human breast cancer cells in culture (Guthrie *et al.* 1997, 1998). In the present experiments we tested deacetyllnomilin, deoxylimonin, ichangin, isoobacunoic acid, limonol, limonin carboxymethoxime, limonin methoxime, methyl deoxylimonate, methyl nomilinate, methyl deacetylnomilinate, methyl isolimonate, nomilin glucoside, nomilinic acid glucoside, 7a-obacunol, obacunone and obacunone glucoside for their ability to inhibit the proliferation of MDA-Mb-435 ER- and MCF-7 ER+ human breast cancer cells. In ER- cells, limonin methoxime and deacetylnomilin were the most effective inhibitors having IC_{50}'s of 0.02 and 0.07 mg/mL respectively. In ER+ cells, deacetylnomilin, obacunone and methyl nomilinate were the most effective inhibitors of proliferation having IC_{50}'s of 0.005, 0.009 and 0.01 mg/mL respectively. These results indicate that citrus limonoids are very potent anti-cancer agents. Studies investigating the effects of citrus limonoids on the growth and metastasis of these cells following their injection into nude mice are now in progress.

calabst.ng

Proceedings Canadian Breast Cancer Research Initiative
Reasons for Hope
Toronto, ON
June 17-19, 1999

THE EFFECTIVENESS OF CITRUS JUICE COMPONENTS IN THE INHIBITION OF HUMAN BREAST CANCER PROLIFERATION

Dionne P. White[1], Najla Guthrie[2], Ted Vandenberg[3], and David J. Freeman[1]

Departments of Pharmacology and Toxicology[1], Biochemistry[2] and Oncology[3], The University of Western Ontario and The Robarts Research Institute, London, ON, N6A3K7, Canada.

Citrus juices have chemical components, which our laboratory has shown to have anticancer properties. The principle agents investigated were the citrus bioflavonoids and the limonoids. The bioflavonoids, nobiletin, tangeretin, hesperetin and naringenin were found to be potent inhibitors of cellular proliferation in both estrogen receptor-positive (ER+) and –negative (ER-) breast cancer cell lines. They were also found to act independently of the estrogen receptor. *In vivo* experiments were performed to determine the ability of citrus bioflavonoids to prevent the establishment and growth of tumors in the nude mouse model. Mice given citrus juices for an eleven-week period, *ad libitum*, developed fewer and smaller tumors than those given diets containing pure bioflavonoid or vehicle (control) only. Compared to controls tumor incidence was less than 50%. Orange juice, followed by grapefruit juice, was the most effective inhibitor of mammary tumor growth. The greater inhibitory actions of the juices may be a result of the combined actions of their constituent flavonoids and limonoids with other components such as vitamin C and hydroxy cinnamic acids. In comparison, the citrus limonoids limonin, nomilin, and a limonoid glucoside mixture were more potent inhibitors of ER- cancer cell proliferation. The maximum tolerated dose, as a percentage of diet was found for each of the limonoids was found and experiments involving nude mice are currently underway to determine the ability of the citrus limonoids to prevent tumor growth and metastasis. The mechanism of action of both sets of compounds is also being studied.

ACS SYMPOSIUM SERIES 758

Citrus Limonoids

Functional Chemicals in Agriculture and Food

Mark A. Berhow, EDITOR
U.S. Department of Agriculture

Shin Hasegawa, EDITOR
U.S. Department of Agriculture

Gary D. Manners, EDITOR
U.S. Department of Agriculture

Chapter 12

Inhibition of Human Breast Cancer Cells by Citrus Limonoids

N. Guthrie[1], K. Morley[1], Shin Hasegawa[2], Gary D. Manners[2], and T. Vandenberg[3]

Departments of [1]Biochemistry and [3]Oncology, The University of Western Ontario, London, Ontario N6A 5C1, Canada
[2]Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, CA 94710

Citrus limonoids are a class of chemically related compounds present in lemon, lime, orange and grapefruit. We have shown that nomilin and a limonoid glucoside mixture are potent inhibitors of prolifertation of estrogen receptor-negative (ER-) and –positive (ER+) human breast cancer cells in culture. In the present experiments, we tested deacetylnomilin, deoxylimonin, ichangin, isoobacunoic acid, limonol, limonin carboxymethoxime, methyl deoxylimonate, methylnomilinate methyl deacetylnomilinate, methyl isolimonate, nomilin glucoside, nomilinic acid glucoside, 7a-obacunol, obacunone and obacunone glucoside for their ability to inhibit the proliferation of MDA-MB-435 ER- and MCF-7 ER+ human breast cancer cells. In ER- cells, limonin methoxime and deacetylnomilin were the most effective inhibitors having IC50s of 0.02 and 0.07 ug/mL respectively. In ER+ cells, deacetylnomilin, obacunone and methyl nomilinate were the most effective inhibitors of proliferation having IC50s of 0.005, 0.009 and 0.01 ug/mL respectively. Maximum tolerated dose studies were conducted in nude mice for limonin and the glucoside mixture. We found that this dose was 2% of the diet for limonin and 4% of the diet for the glucoside mixture. These results suggest that citrus limonoids have important anti-cancer activity.

Breast cancer is the second most common cause of cancer related deaths in North American women (1). Modern preventative and treatment methods are limited and the investigation of natural, low toxicity dietary components for their anti-cancer properties is of great interest. There is general

agreement that plant-based diets rich in whole grains legumes, fruits and vegetables, reduce the risk of various types of cancer, including breast cancer, and a variety of compounds produced by plants have been investigated for their anti-cancer activity (2-7). Our results have shown that citrus flavonoids inhibit the proliferation of ER- and ER+ human breast cancer cells *in vitro* (8). In addition to the *in vitro* studies, we have previously reported that giving orange juice or naringin (the glycoside form of the flavonoid, naringenin, in grapefruit) to rats delayed the development of mammary tumors induced by 7,12-dimethylbenz(a)anthracene (DMBA) (8). In a more recent experiment, MDA-MB-435 ER- human breast cancer cells were injected into the mammary fat pads of nude mice. Giving the animals orange juice or grapefruit juice instead of water was found to reduce the incidence of tumors at the site of injection by more than 50% and to inhibit markedly metastases to the lymph nodes and lungs (9). The constituent flavonoids from orange or grapefruit juice appeared to be less effective inhibitors of cancer development and metastases in this experiment. Both orange and grapefruit juice contain other bioactive components (Table 1).

Table 1. Average Amounts of Bioactive Compounds in Orange Juice and Grapefruit Juice (mg/L).

Component	*Orange Juice*	*Grapefruit Juice*
Hesperidin	205	85
Naringin	18	246
Methoxylated flavones	6	<5
Limonene	330	330
Limonoid glucosides	366	198
Limonin glucoside	209	137
Limonin	3	10
Total carotenoids	18	59
Hydroxycinnamic acids	80	89
Ascorbic Acid (Vit. C)	284	250

These include the limonoids, which are one of the two bitter principles found in citrus fruits, including oranges, grapefruits, lemons and limes (10-12). They are also present as glucose derivatives in mature fruit tissues and seeds, and are one of the major secondary metabolites present in citrus. Citrus limonoids were observed to inhibit the proliferation of human breast cancer cells more effectively than the flavonoids (9) and may be largely responsible for the anti-cancer effects of the juices.

Our interest in limonoids began with the observation that orange and grapefruit juice inhibited the growth and metastases of human breast cancer

cells injected into the mammary fat pad of nude mice and that this inhibition was not completely due to their constituent flavonoids (9). Limonoids have been shown to have anti-cancer activity (13-16). Nomilin reduced the incidence of and number of chemically-induced forestomach tumors in mice when given by gavage (14). Addition of nomilin and limonin to the diet inhibited lung tumor formation in mice and topical application of the limonoids was found to inhibit both the initiation and promotion phases of carcinogenesis in the skin of mice (15).

In the present experiments, we investigated a number of naturally-occurring and synthetic limonoids for their ability to inhibit the proliferation of MDA-MB-435 ER- and MCF-7 ER+ human breast cancer cells in culture. We have also conducted studies in animals to determine the maximum tolerated dose for limonin and the limonoid glucoside mixture.

Citrus Limonoids against Human Breast Cancer Cells in Culture

Cell Culture

MDA-MB-435 estrogen receptor-negative human breast cancer cells were maintained at 37°C in minimum essential medium (alpha modification) containing 3.7 g of sodium bicarbonate per litre, supplemented with 10% v/v fetal calf serum and 1% v/v fungizone (antibiotic/antimycotic, 10 000 units/mL penicillin G sodium, 10 000 µg/mL streptomycin sulphate and 25 µg/mL amphotericin B in 0.85% saline), in a humidified atmosphere of 5% carbon dioxide. Stock cultures were seeded at a density of 2 x 10^5 cells and allowed to multiply for 48-72 hours.

MCF-7 estrogen receptor-positive human breast cancer cells were maintained in minimum essential medium (alpha modification) containing 3.7 g of sodium bicarbonate supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 10 µg/mL insulin and 1% v/v fungizone (antibiotic/antimycotic, 10 000 units/mL penicillin G sodium, 10 000 µg/mL streptomycin sulphate and 25 µg/mL amphotericin B in 0.85% saline). Cells were grown to confluence at 37°C in a humidified atmosphere containing 5% carbon dioxide and were passaged weekly using 0.25% trypsin.

Experiments on cell proliferation

The effects of each of the limonoids on the proliferation of MDA-MB-435 ER- and MCF-7 ER+ human breast cancer cells were examined by determining the incorporation of [^{3}H] thymidine into growing cells. MDA-MB-435 cells were plated at a density of 2 x 10^4 cells/well in 96-well, flat-bottomed tissue culture plates in a total volume of 200 µL of medium and incubated at 37°C, with or without the test compounds. The plates were incubated for 48 hours at 37°C and [^{3}H] thymidine was then added to determine the number of dividing cells at each concentration. The cells were reincubated for 4 hours, after which the medium and excess radiolabel were removed. The cells were trypsinized and harvested onto a glass fiber filter paper, and the radioactivity was counted. The percentage of dividing cells

was determined by comparing the number of disintegrations per minute of the treated cells (average of 3 wells/concentration) with that obtained for the control cells. The concentrations at which 50% growth inhibition occured was determined (IC_{50}) for each compound (17).

We initially tested the effects of limonin, nomilin, limonin glucoside, and a glucoside mixture (limonin-30%, nomilinic acid-33.8%, nomilin-12.7%, obacunone-8.2%, deacetylnomilin-6.6%, deacetylnomilinic acid-8.7%) from orange molasses on the proliferation of MDA-MB-435 ER- and MCF-7 ER+ cells. The IC_{50} values for each limonoid are given in Tables 2 and 3.

Table 2: Effect of Limonoids on the Proliferation and Viability of MDA-MB-435 Estrogen Receptor-Negative Human Breast Cancer Cells in Culture.

Limonoid	*IC_{50} (μg/mL)*	*LC_{50} (μg/mL)*
Limonin glucoside	75	500
Limonin	12	80
Nomilin	0.4	3
Glucoside mixture	0.08	6

Table 3: Effect of Limonoids on the Proliferation and Viability of MCF-7 Estrogen Receptor-Positive Human Breast Cancer Cells in Culture.

Limonoid	*IC_{50} (μg/mL)*	*LC_{50} (μg/mL)*
Limonin glucoside	35	125
Limonin	2	63
Nomilin	0.05	2
Glucoside mixture	0.05	4

In ER- cells, the most potent inhibitor was the glucoside mixture having an IC_{50} of 0.08 ug/mL followed by nomilin and limonin. Limonin glucoside was the least effective having an IC_{50} of 75 ug/mL. The limonoids inhibited ER+ cells more effectively (Table 3). Both nomilin and the glucoside mixture were the most effective having an IC_{50} of 0.05ug/mL (Table 3). We have

also tested the effects of a number of naturally occurring and synthetic limonoids for their effect on cell proliferation. In ER- cells, limonin methoxime and deacetylnomilin were the most effective inhibitors having IC_{50}s of 0.02 and 0.07 ug/mL respectively (Table 4, Figure 1). In ER+ cells, deacetylnomilin, obacunone and methyl nomilinate were the most effective inhibitors of proliferation having IC_{50}s of 0.005, 0.009 and 0.01 µg/mL respectively (Table 5, Figure 2). Tamoxifen, a drug widely used in the treatment of hormone responsive breast cancers, acts mainly by competing with estrogen for its receptor. The IC50s for tamoxifen were 90 µg/mL in ER- cells and 0.04 µg/mL in ER+ cells (Tables 4 and 5). Our data indicate that citrus limonoids are potent inhibitors of both cell types and may act via an estrogen receptor-independent pathway. Nomilin and the glucoside mixture are comparable in their inhibition of MCF-7 cells to tamoxifen. However, deacetylnomilin, obacunone and methyl nomilinate are more effective in inhibiting ER+ cells than tamoxifen (Table 4).

Table 4: Effect of Limonoids on the Proliferation of MDA-MB-435 Estrogen Receptor-Negative Human Breast Cancer Cells in Culture.

Limonoid	*IC_{50} (µg/mL)*
Limonin methoxime	0.02
Deacetylnomilin	0.07
Deoxylimonin	0.78
Nomilin glucoside	0.78
Isoobacunoic acid	1.95
7-a obacunol	3.13
Ichangin	3.13
Limonol	3.13
Obacunone	3.13
Nomilinic acid glucoside	3.13
Obacunone glucoside	6.25
Limonin carboxymethoxime	10.3
Methyl deacetylnomilinate	12.5
Methyl deoxylimonate	25.0
Methyl nomilinate	25.0
Methyl isolimonate	25.0
Tamoxifen	90.0

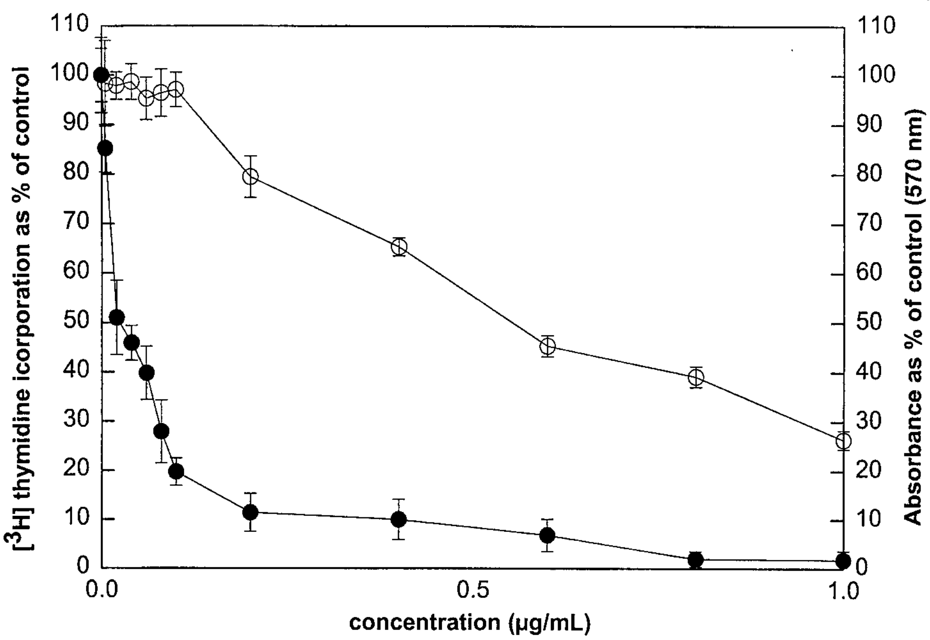


Figure 1. Effect of limonin methoxime on the proliferation (•) and survival (0) of MDA-MB-435 estrogen receptor-negative human breast cancer cells in culture as determined by the incorporation of [^{3}H] thymidine and MTT respectively. Results are the averages of 3 separate experiments ±SEM.

TABLE 5: Effect of Limonoids on the Proliferation of MCF-7 Estrogen Receptor-Positive Human Breast Cancer Cells in Culture.

Limonoid	*IC_{50} (µg/mL)*
Deacetylnomilin	0.005
Obacunone	0.009
Methyl nomilinate	0.01
7-a obacunol	0.15
Nomilin glucoside	0.78
Isoobacunoic acid	0.78
Nomilinic acid glucoside	1.95
Obacunone glucoside	1.95
Limonin carboxymethoxime	2.50
Deoxylimonin	2.50
Methyl deacetylnomilinate	2.95
Ichangin	3.13
Limonin methoxime	3.13
Limonol	6.25
Methyl deoxylimonate	12.5
Methyl isolimonate	12.5
Tamoxifen	0.04

Experiments on Cell Growth

The effect of limonoids on the growth of both types of cells was also studied. MDA-MB-435 and MCF-7 cells were plated at 1 x 10^4 cells/dish in 60 mm dishes, with or without the test compounds at their IC_{50} concentration (determined in the proliferation assays) in a total volume of 7 mL. The cells were removed by trypsinization at the specified times and counted using a hemocytometer (17).

The ability of limonoids to suppress MDA-MB-435 and MCF-7 cell growth was evident when the cells were grown in the presence of the limonoids at their IC50 values for 10 days, as illustrated for nomilin in Figure 3. The effects were clearly apparent after 2 days of treatment, but cell growth was impeded over the entire period of 10 days.

Animal Studies

Maximum Tolerated Dose

Groups of nude mice NCR nu/nu were maintained in a pathogen free animal facility. After a period for acclimitization, they were placed on test diets containing limonin or a glucoside mixture at the initial level of 0.5% by

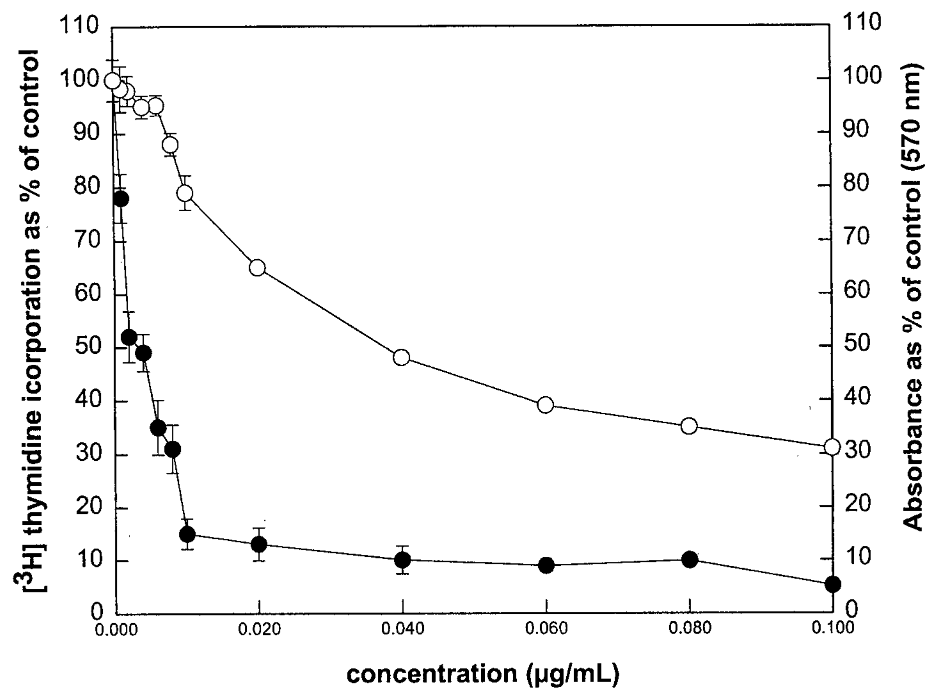


Figure 2. Effect of deacetylnomilin on the proliferation (•) and survival (0) of MCF-7 estrogen receptor-positive human breast cancer cells in culture as determined by the incorporation of [^{3}H] thymidine and MTT respectively. Results are the averages of 3 separate experiments ±SEM.

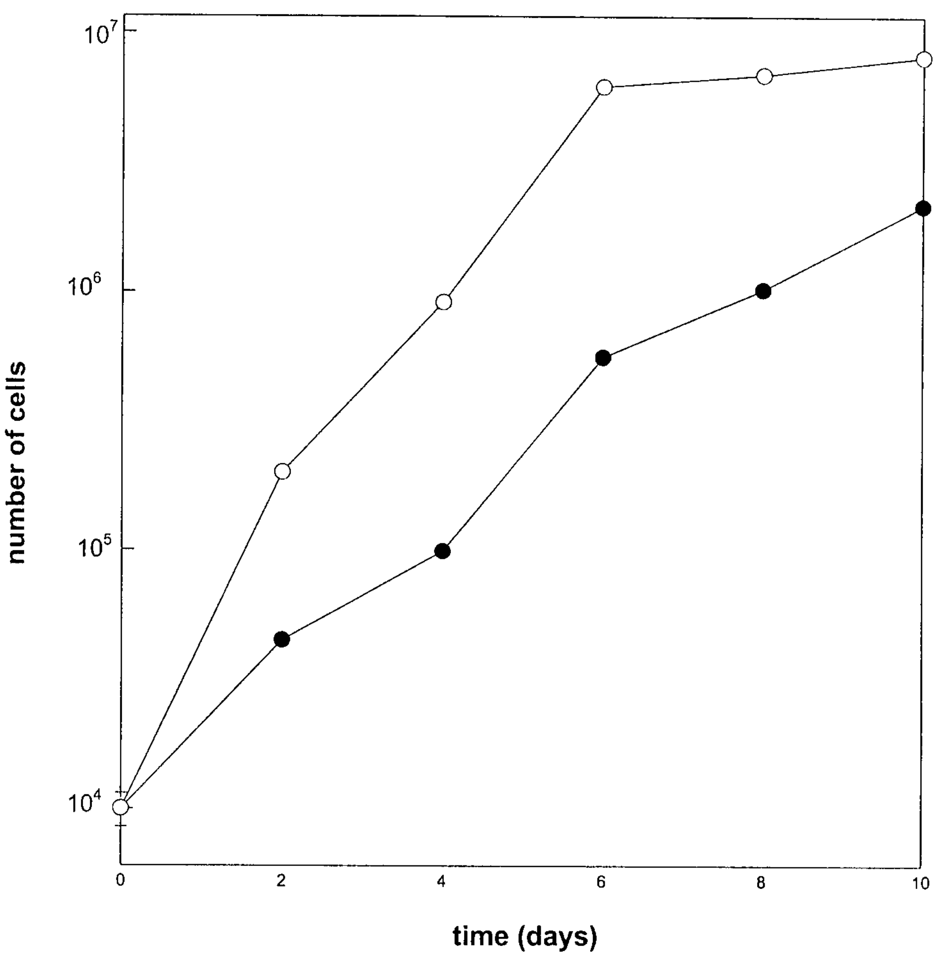


Figure 3. Growth of MDA-MB-435 estrogen receptor-negative human breast cancer cells in presence (•) and absence (0) of nomilin at the concentration that inhibited cell proliferation by 50%. Results are the averages of 3 separate experiments ±SEM.

weight. The animals were monitered daily for changes in weight, activity, mobility, and overall health. They were observed for 2 weeks and if no signs of toxicity were observed the level of limonoids in the diet was doubled for other groups of animals. This doubling was repeated until signs of toxicity appeared. The dose ranged from 0.5% to 8% for limonin or the glucoside mixture by weight. At autopsy, liver samples were examined histologically for signs of toxicity.

No significant weight loss was observed for the glucoside mixture at the 8% level. However, histological examination of the liver samples showed signs of early degeneration at 8% and 4% levels. The groups receiving limonin exhibited weight loss and signs of inactivity at the 8% level. Also, liver toxicity was observed at 4% levels for limonin. Based on these findings, we concluded that the maximum tolerated dose is 2% by weight for limonin and 4% by weight for the glucoside mixture. It is also important to note that the duration for these studies was short due to availability of limonoids.

Summary and Conclusions

Our results have shown that orange juice and grapefruit juice inhibit the proliferation and metastases of MDA-MB-435 ER- human breast cancer cells injected into nude mice and that this inhibition is only partially due to their constituent flavonoids. Limonoids are also present in the juices. We have recently reported limonoids to be more potent inhibitors of these cells than the flavonoids *in vitro*. We have also shown that citrus limonoids are effective at inhibiting MCF-7 ER+ human breast cancer cells in culture with nomilin and the glucoside mixture having the lowest IC50s. In testing other naturally-occurring and synthetic limonoids, we found that limonin methoxime and deacetylnomilin were the most effective inhibitors of proliferation in ER- cells and deacetylnomilin, obacunone and methyl nomilinate were the most effective inhibitors of proliferation in ER+ cells. In addition, when citrus limonoids were compared to tamoxifen, nomilin and the glucoside mixture inhibited ER+ cells to the same extent, whereas deacetylnomilin, obacunone and methyl nomilinate were much more effective. *In vivo*, we determined the maximum tolerated dose for limonin (2%) and the glucoside mixture (4%).

The inhibition of both ER- and ER+ cells by these limonoids has important clinical implications, since most breast cancers are heterogeneous and consist of both cell types. Tamoxifen is effective in arresting the growth of ER+ cells and thus is widely used in the treatment of hormone-responsive tumors, but is relatively ineffective in treating ER- tumors. Therefore, an alternative treatment regimen, using a growth inhibitor of ER-, coupled with an anti-hormonal drug would effectively target both types of cells. As normal constituents of food, the limonoids are relatively non-toxic and may significantly reduce the risk of cancer if ingested on a regular basis. They are also relatively inexpensive compared to other drugs used in chemotherapy. The limonoids could thus prove to be an important new means of treatment and/or prevention of breast cancer.

References

1. Pisani, P. J. *Environ. Pathol. Toxicol. Oncol.* **1992,** *11*, 313.
2. Steinmetz, K.A.; Potter, J.D. *Cancer Causes Control* **1991,** *2*, 325.
3. Steinmetz, K.A.; Potter, J.D. *Cancer Causes Control* **1991,** *2*, 427.
4. Block, G.; Patterson, B.; Subar, A. *Nutr. Cancer* **1992**, *18*, 1.
5. Wattenberg, L.W. *Cancer Res.* **1992**, *52*, 2085.
6. Willett, W.C. *Environ. Health Perspect.* **1995**, *103*, 165.
7. American Institute for Cancer Research. *Adv. Exp. Med. Biol.* **1996,** *401*, 1.
8. So, F.V.; Guthrie, N.; Chambers, A.F.; Moussa, M.; Carroll, K.K. *Nutr. Cancer* **1996,** *26*, 167.
9. Guthrie, N.; Carroll, K.K. In *Biological Oxidants:Molecular Mechanisms and Health Effects;* Packer, L.; Ong, A.S.H., Eds.; AOCS Press, Champaign, IL, 1998, p 310.
10. Fong, C.H.; Hasagawa, S.; Herman, Z.; Ou, P. *J. Food Sci.* **1989,** *54*, 1505.
11. Hasegawa, S.; Herman, Z. In *Secondary–Metabolite Biosynthesis and Metabolism;* Petroski, R.J.; McCormick, S.P., Eds.; Plenum Press, New York, 1992, p 305.
12. Hasegawa, S.; Miyake, M. *Food Rev. Int.* **1996,** *12*, 413.
13. Lam, L.K.T. *Carcinogenesis* **1989,** *10*, 1535.
14. Lam, L.K.T.; Hasegawa, S. *Nutr. Cancer* **1989,** *12*, 43.
15. Lam, L.K.T.; Zhang, J.; Hasegawa, S.; Herman, A.J.S. In *Food Phytochemicals for Cancer Prevention I: Fruits and Vegetables;* Huang, M-T.; Osawa, T.; Ho, C-T.; Rosen, R.T., Eds.; Am. Chem. Soc., Washington, DC, 1994, p 209.
16. Miller, E.G.; Gonzales-Sanders, A.P.; Couvillon, A.M.; Wright, J.M.; Hasegawa, S.; Lam, L.K.T.; Sunahara, G.I. In *Food Phytochemicals for Cancer Prevention I: Fruits and Vegetables.* Huang, M-T.; Osawa, T.; Ho, C-T.; Rosen, R.T., Eds.; Am. Chem. Soc., Washington, DC, 1994, p 220.
17. Kothapalli, R.; Guthrie, N.; Chambers, A.F.; Carroll K.K. *Lipids* **1993**, *28*, 969.
18. Hansen, M.B.; Nielson, S.E. *J. Immunol. Methods* **1989,** *119*, 203.

Reprinted from ACS Symposium Series 758
Citrus Limonoids
Functional Chemicals in Agriculture and Food
M.A. Berhow, S. Hasegawa, G.D. Manners, Editors
Published 2000 by the American Chemical Society

Please select Print from the file menu to print your Abstract.

Presenting Author: Theodore A Vandenberg BA, MD
Category: 13. Diet and Nutrition

24th Annual San Antonio Breast Cancer Symposium Abstract Submission Printout

Filename: 550713
Contact Author: Theodore A Vandenberg MD
Department/Institution: Oncology, London Regional Cancer Centre
Address: 790 Commissioners Rd. E., London, Ontario, Canada, N6A 4L6
Phone: 519-685-8640 **Fax:** 519-685-8624 **E-Mail:** ted.vandenberg@lrcc.on.ca
Presentation Preference No preference
Do not withdraw my abstract if my preference cannot be accomodated.
Award: No
Permission to Reproduce Presentation: I agree to all recording and reproduction described.
Scholars Program: No **Postdoctoral type:** NA

Inhibition of human beast cancer cell growth in nude mice by citrus limonoids.
Theodore A Vandenberg*, Shin Hasegawa, Gary D Manners and Najla Guthrie. Oncology, University of Western Ontario, London, Ontario, Canada; Western Regional Research Center, USDA, Albany, CA; Western Regional Research Center, USDA, Albany, CA, United States; and Biochemistry, University of Western Ontario, London, Ontario, Canada.

Citrus limonoids are one of the two bitter principles found in citrus fruits, such as lemon, lime, orange and grapefruit.They have been shown to have anti-cancer activity both in vitro and in vivo. The following studies examine the ability of citrus limonoids to slow or reverse the growth of previously established tumours and their ability to prevent regrowth of resected tumours in mice. Estrogen receptor-negative human breast cancer cells were injected into the mammary fat pads of nude mice. In the first study, treatment with limonoids began 4 weeks following injections. In the second study, treatment with limonoids began following the surgical removal of the tumors. We found that limonin and the glucoside mixture slowed the growth of already established tumors and they were also the most effective in preventing recurrence of mammary fat pad tumors following resection. In studies investigating the mechanism of action, limonin was observed to increase the percentages of cells in the G2M phase followed by nomilin and the glucoside mixture. These results suggest that citrus limonoids may modulate cellular proliferation in MDA-MD-435 ER- human breast cancer cells by causing an accumulation of cells in the G2M cell cycle phase.

Disclosure: N Guthrie, President KGK Synergize

Signature: ______________________
(Theodore A Vandenberg ~~BA, MD~~), Presenting Author